Modulation of global stability, ligand binding and catalytic properties of trypsin by anions

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Abstract

Specific salts effect is well-known on stability and solubility of proteins, however, relatively limited knowledge is known regarding the effect on catalytic properties of enzymes. Here, we examined the effect of four sodium anions on thermal stability and catalytic properties of trypsin and binding of the fluorescent probe, p-aminobenzamidine (PAB), to the enzyme. We show that the specific anions effect on trypsin properties agrees with the localization of the anions in the Hofmeister series. Thermal stability of trypsin, T_m, the affinity of the fluorescent probe to the binding site, K_d, and the rate constant, k_{cat}, of trypsin-catalyzed hydrolysis of the substrate N-benzoyl-L-arginine ethyl ester (BAEE) increase with increasing kosmotropic character of anions in the order: perchlorate<bromide<chloride<sulfate, while the value of Michaelis constant, K_M, decreases. Correlations between the values of T_m, K_d for PAB, k_{cat}, and K_M for BAEE in the presence of 1M studied salts suggest interrelation among these parameters of the enzyme. Global stabilization as well as increased rigidity of trypsin is accompanied by strengthening of interaction with fluorescent probe PAB and in accordance with decreasing values of K_M for the substrate BAEE. Strong correlations between parameters characterizing the trypsin properties with the charge densities of anions clearly indicate direct electrostatic interaction as a basis of the specific anion effect on the conformational and functional properties of the enzyme.

Key words: Hofmeister effect; macromolecular rate theory; enzyme dynamics; enzyme activity; serine protease

Introduction

Ions are part of all biological systems and an understanding of ions interaction at physiological conditions with macromolecules has been devoted to numerous works. Specific ions effect also known as the Hofmeister effect deals with the effect of ions on biological systems at high (> 0.3 M) salt concentrations. It has been shown that the specific ions effect influence numerous fundamental properties of proteins such as conformational equilibrium between different states of proteins [1–4], protein dynamics/flexibility [5–9], protein stability [10–14], and formation of amorphous aggregates and ordered fibrils [15–17]. Salts modulation of equilibrium between conformational states by decreasing a barrier between them inevitably increases the rate of interconversion (dynamics) as well as the accessibility of these two states (flexibility) [3,8]. As it has been shown previously, modification of the energy landscape of the active site affects substrate binding and rate of the substrate transformation by the enzyme [13,14,18–24].

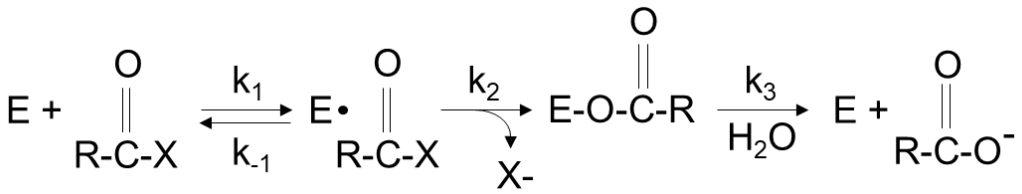
Modulation of enzyme properties by high ionic strength of particular ions is attractive also for utilization of such conditions in biotechnological processes in the effort to diminish bacterial contamination of reaction vessels and thus significantly reduce expenses related to sterilization and the necessity to employ expensive materials for fermentation facilities [25]. Moreover, understanding of particular specific salts effect may lead to exotic implications regarding the habitability of extraterrestrial bodies such as Mars due to a discovery of increased concentrations of salts (sulfates and perchlorates) at and beneath the surface of Mars [26–28].

The Hofmeister effect on catalytic properties of chymotrypsin, the member of the large superfamily of serine proteases [29], has been recently systematically studied [14,24]. In the present study, to demonstrate the universal nature of the specific salts effect we chose trypsin, the enzyme homologous to chymotrypsin. Trypsin and chymotrypsin are composed of two-six stranded anti-parallel β-barrel domains with superimposable main chain structures. The primary substrate-binding site, called the S1 binding pocket, is formed by residues #189-195, #214-220 and #226-228. Trypsin preference for lysine and arginine results from the presence of Asp189 (Ser in the case of chymotrypsin) at the bottom of the S1 binding pocket. Interestingly, mutation Asp189Ser in trypsin does not switch the substrate specificity of trypsin to that of chymotrypsin [30] but to reach the conversion of trypsin to chymotrypsin-like protease also the exchange of flexible surface loops L1 and L2 formed by residues #185-188 and #221-225, respectively, is unavoidable. Noteworthy, the loops L1 and L2 are not structural components of either S1 binding site or the extended substrate binding site [31]. This suggests a significant role of dynamics/flexibility of the enzyme both for substrate recognition and the catalytic rate. In fact,

it seems that the dynamics/flexibility of the polypeptide chain has been conserved not only in serine proteases [32,33] but in enzymes in general [34,35].

Recently developed macromolecular rate theory (MMRT) addresses a role of dynamics for enzyme catalysis. MMRT is based on observation that enzyme-catalyzed reactions occur with significant negative value of the heat capacity, which is consistent with a classical description of enzyme catalysis, i.e. that the enzyme binds relatively weakly to the substrate but very tightly to the transition state [36]. Interestingly, it has been demonstrated that to the reduction of the heat capacity change contribute not only the active site region but also domains distal to the active [37] suggesting thus a role of the whole enzyme for the catalysis.

From a mechanistic point of view, the reaction process of serine proteases consists of three steps (Scheme 1): (i) substrate binding - formation of enzyme-substrate (k_1, k_{-1}), (ii) acylation step – formation of covalent adduct (k_2), and (iii) deacylation step restoring the original state of the enzyme (k_3) [38].



Scheme 1. The kinetic mechanism of the serine protease reaction. This enzymatic reaction is described by the parameters of $k_{cat}=k_2k_3/(k_2+k_3)$ and $K_M=K_dk_3/(k_2+k_3)$, where K_d is dissociation constant, which equals k_{-1}/k_1.

In the present work, for analysis of the specific anions effect on trypsin properties have been chosen four sodium anions from different parts of the Hofmeister series: perchlorate, bromide, chloride, and sulfate. We show that specific anions effect influence global stability and polypeptide chain rigidity/flexibility as well as local properties of the binding site of the trypsin. Destabilization of the trypsin, although with the transition temperature still above the room temperature, is accompanied by a decrease in the affinity of the enzyme to a small ligand and a decrease in the catalytic rate of the substrate hydrolysis. Analysis of the dependences of enzyme parameters, such as the catalytic rate, the Michaelis constant, the dissociation constant of the ligand, and the transition temperature, on the anions charge density, implicates that the anions specific effect is intermediated through electrostatic interactions.

Materials and Methods

Enzyme trypsin (EC 3.4.21.4) from bovine pancreas, N-benzoyl-L-arginine ethyl ester (BAEE), HCl, $Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, NaCl, $NaClO_4.H_2O$, NaOH and p-aminobenzamidine were purchased from Sigma Aldrich. NaBr from Penta, Na_2SO_4 from Merck, and PeFabloc and protective solution PeFabloc were purchased from Roche.

Modification of trypsin. Trypsin was modified by PeFabloc© SC, covalent inhibitor of the class of sulfonyl fluorides, to prevent possible autolysis during thermal denaturation experiments. In all other experiments, trypsin was used without the modification by PeFabloc© SC. The reaction mixture, based on the manufacturer's manual, contained 500 μL of chymotrypsin and 30 mg of PSC-protector added to 1 mg of PeFabloc© SC and incubated 1 hour at room temperature. After 1 hour incubation, the mixture has been dialyzed through dialyzed membrane with molecular weight cut-off 14000 Da in 20 mM phosphate buffer at 4°C overnight and used for thermal stability measurements.

Circular dichroism measurements. Circular dichroism (CD) spectra of trypsin in the far-UV spectral region were performed with spectropolarimeter Jasco J-815 using a 1 mm path-length quartz cuvette at 25°C. The temperature was controlled with a Peltier block CDF-426S/15. The spectra are the average of 8 consecutive scans, recorded with a scan rate of 50 nm/min and bandwidth of 1 nm. The protein concentration was 30 μM. Measurements were performed in 20 mM phosphate buffer with pH 7.6, in the absence and in the presence of 1 M salts.

Thermal stability of modified trypsin. The measurements of thermally induced transitions of modified trypsin were performed by spectropolarimeter Jasco J-815 by monitoring the ellipticity at a single wavelength from the far-UV region by circular dichroism. The temperature, controlled with Peltier block CDF-426S/15, increased from 20°C to 90°C and data were recorded at a scan rate of 1.5°C/min. Measurements were performed in 20 mM phosphate buffer and 1M salts ($NaClO_4$, NaCl and Na_2SO_4) by monitoring the CD signal at 210 nm and due to the high absorbance of NaBr in the UV area below 230 nm, the CD signal was monitored at 235 nm. Trypsin concentration was 50 μM in the absence (20 mM phosphate buffer) and in the presence of 1M salts with pH 7.6.

Thermal denaturation was analyzed by fitting experimentally obtained data according to the following equation:

$$y_{obs} = \frac{y_N + y_D . \exp\left[\frac{\Delta H_{vH}}{R} . \left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}{1 + exp\left[\frac{\Delta H_{vH}}{R} . \left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}$$

(Equation 1)

where y_{obs} is experimentally measured parameter, y_N is the value of the measured parameter for native state, y_D is the value of measured parameter for denaturated state, ΔH_{vH} is the van´t Hoff enthalpy change, R is the gas constant and T_m is the transition temperature.

Fluorescent probe binding into the active site of trypsin. The emission measurements of fluorescent probe (p-aminobezamidine) binding into the active site of trypsin were carried out with Varian Cary Eclipse fluorescence spectrophotometer using 1 cm path-length quartz cuvette at 25°C. The fluorescent emission spectra in the range of 330 nm to 430 nm were obtained after excitation with 325 nm.

In the first set of measurements, we observed the change in fluorescent intensity at 363 nm due to the increasing concentration of p-aminobenzamidine (PAB) in 20 mM phosphate and 1M salts ($NaClO_4$, NaCl, NaBr and Na_2SO_4). Protein concentration was 5 µM and the concentration of p-aminobezamidine changed from 1.6 µM to 46 µM. The PAB fluorescence in the presence of trypsin was corrected for PAB in the corresponding conditions in the absence of the protein. The dependences of the amplitude of maximum fluorescence on the concentration of p-aminobezamidine were analyzed by the followed equation:

$$\frac{[RL]}{[R]} = \frac{\left(1 + \frac{[L]}{[R]} + \frac{K_d}{[R]}\right) - \sqrt{\left(1 + \frac{[L]}{[R]} + \frac{K_d}{[R]}\right)^2 - 4.\frac{[L]}{[R]}}}{2}$$

(Equation 2)

where [RL]/[L] is the fraction of occupied binding sites and it corresponds with experimentally measured value of fluorescence, [R] is the concentration of enzyme, [L] is the concentration of ligand (PAB) and K_d is dissociation constant of ligand from an enzyme. Based on this equation we determined the dissociation constant, K_d and the maximum fluorescence, $F_{max,}$ of the fluorescence probe in the presence of 1 M studied salts.

In the second set of measurements, we observed a change in fluorescent intensity at 363 nm due to the increasing concentration of salts from 0.1 M to 1.2 M. The sample contained the 40 µM of p-aminobezamidine dihydrochloride and 5 µM trypsin.

Measurements of kinetic parameters of trypsin. Measurements for determination of catalytic activity of trypsin were performed by UV-Vis absorption spectrophotometer Specord S300 equipped by Peltier block Analytic Jena in 1 cm path-length quartz cuvette. The concentration of trypsin was determined using an extinction coefficient of 38,400 $M^{-1}.cm^{-1}$ at 280 nm. Trypsin catalyzed hydrolysis of substrate, BAEE, was measured by following the increase in absorbance at 253 nm for 2 minutes and the data were collected every 3 seconds.

The reaction mixture of kinetic measurements contained corresponding concentration (0.25 M, 0.5 M, 0.75 M and 1 M) of $NaClO_4$, NaCl, NaBr and Na_2SO_4 and 44.2 nM trypsin in 20 mM phosphate buffer with pH 7.6. The appropriate volume of BAEE (concentration range from 2 µM to 420 µM) was added to start the enzymatic reaction. The reaction mixture with the enzyme was incubated at 25°C for 4 minutes before each measurement. The initial reaction velocities were determined from the slope of the initial increase in the absorbance at the corresponding wavelength. The absorbance was converted to molar concentration using the molar extinction cofficient for N-benzoyl-L-arginine (cleavage product of BAEE) of 880 $M^{-1}.cm^{-1}$ at 253 nm.

Experimentally determined data were fitted according to Michaelis-Menten equation:

$$v = \frac{V_{max} \cdot [S]}{K_M + [S]}$$

(Equation 3)

where V_{max} is the maximal velocity, [*S*] is the substrate concentrations and K_M is the Michaelis constant, the concentration of substrate at which the reaction rate is equal to half of its maximal value.

Molecular dynamics simulations. The GROMACS package [39] was used to carry out the molecular dynamics simulations of systems involving trypsin (EC: 3.4.21.4, RCSB PDB: 4I8H) in pure water and in the aqueous solution of Na_2SO_4, NaCl, and $NaClO_4$ at the concentration of 1M. The AMBER99SB-ILDN force field [40] and potential parameters for proteins were used to describe the interaction in the system. To build the initial structure, the trypsin was placed in the center of the cubic box. Then cations and anions were added randomly by the Packmol package in the simulation boxes [41,42] to reach the desired salt concentrations. Further, the simulations box was solvated by water molecules, using the TIP3P model of water [43]. Energy minimization by using steepest descent followed by equilibration at constant temperature 300 K by NVT (canonical ensemble) and NPT (isothermal–isobaric) restrained simulations. During equilibration velocity-rescaling, temperature coupling (modified Berendsen thermostat) was used [44] for temperature control 300 K and a Parrinello-Rahman barostat [45] for pressure control 1 bar. The MD production was performed for 100 ns at 300K with 2 fs time steps. Visual Molecular Dynamics (VMD) [46] and PyMOL [47] were applied for visualizations and preparation of snapshots.

Results

Global property – secondary structure and thermal stability. The global conformation of proteins is well represented by their secondary structures. From the comparison of trypsin CD spectra in the far-UV spectral region in the absence and the presence of salts, one can exclude a significant effect of 1M salts on the secondary structure of the enzyme (inset Figure 1). As

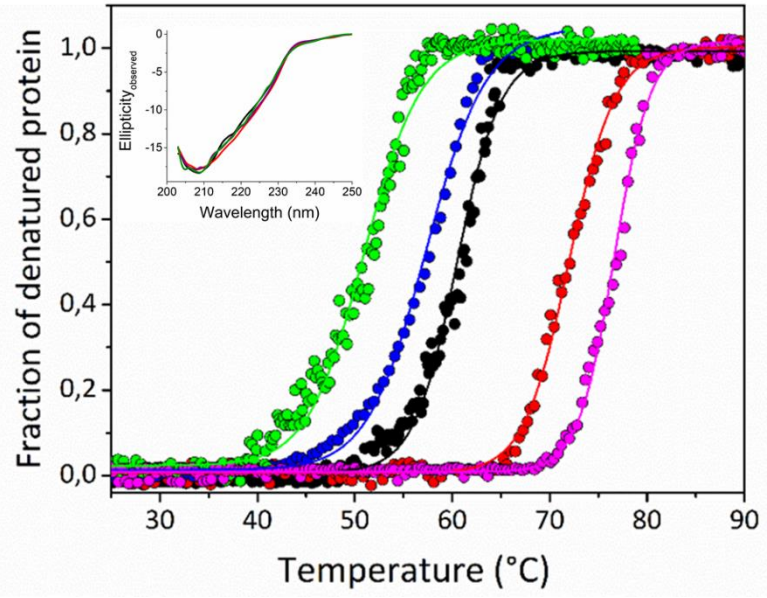


Figure 1. Thermal transitions of trypsin measured in the far-UV CD region in the absence of salts (**black**) and in the presence of 1 M sodium salt; perchlorate (**green**), bromide (**blue**), chloride (**red**), and sulfate (**purple**). The experimental data (symbols) were fitted by Equation 1. Inset: CD spectra of trypsin in the **buffer**, **perchlorate**, **chloride**, and **sulfate**. The CD spectrum of trypsin in the presence of bromide is not shown due to the high absorbance of the bromide anions below 230 nm.

the measure of thermal stability of trypsin in the studied conditions we used transition temperature determined from thermal denaturation. The thermal denaturations of trypsin at given conditions have been followed by CD in the far-UV spectral region (at 235 nm in the case of sodium bromide and 210 nm in the case of other salts) in the presence of 1 M salts. To prevent unspecific autolysis of trypsin during the thermal denaturation measurement, trypsin was inhibited by covalent modification of the serine residue of the catalytic triad by the reagent PeFabloc© SC. Thermal denaturation curves had a sigmoidal shape in all studied conditions (Figure 1). Transition temperatures, T_m, corresponding to the inflexion points of the curves, were obtained from fitting experimental data to Equation 1 (Table 1).

Table 1. Parameters characterizing conformational and functional properties of trypsin (with standard deviations).					
	buffer	1 M Na_2SO_4	1 M NaCl	1 M NaBr	1 M $NaClO_4$
T_m (°C)	60.5 ± 0.1	76.7 ± 0.1	72.0 ± 0.1	57.4 ± 0.1	51.0 ± 0.1
V_{max} (%)	100 ± 2	93 ± 3	76 ± 2	74 ± 2	47 ± 1
K_M (μM)	9.2 ± 1.1	10.2 ± 1.1	15.2 ± 1.7	20.9 ± 2.1	28.9 ± 3.3
F_{max} (a.u.)	100 ± 3	128 ± 3	97 ± 5	87 ± 4	46 ± 4
K_d (μM)	2.5 ± 0.1	3.1 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	6.1 ± 0.1

The effect of anions on the thermal stability agrees with their position in the Hofmeister series and can be ranked according to their destabilization effect as follows: sulfate<chloride<bromide<perchlorate. The excellent fits of the experimental data by Equation 1, which describes temperature-induced two-state conformational transition, suggest an apparent all-or-none transition, i.e. the transition without intermediate(s). In such a case, the transition temperature is a good measure of the global stability of trypsin.

Global and local conformation of trypsin – MD simulations. The trajectories generated from the simulations were analyzed for the root mean square deviation (RMSD) of C-alpha atoms of the trypsin to show the deviations of the backbone atoms of the enzyme from its initial structure. RMSD shows how the structures and parts of structures change over time as compared to the starting point, which comes from the crystal structure with PDB code of 4I8H. RMSD calculations of trypsin in water and 1M aqueous solutions of Na_2SO_4, NaCl, and $NaClO_4$ show that the trypsin enzyme is more stable in the aqueous solution of Na_2SO_4 compared to

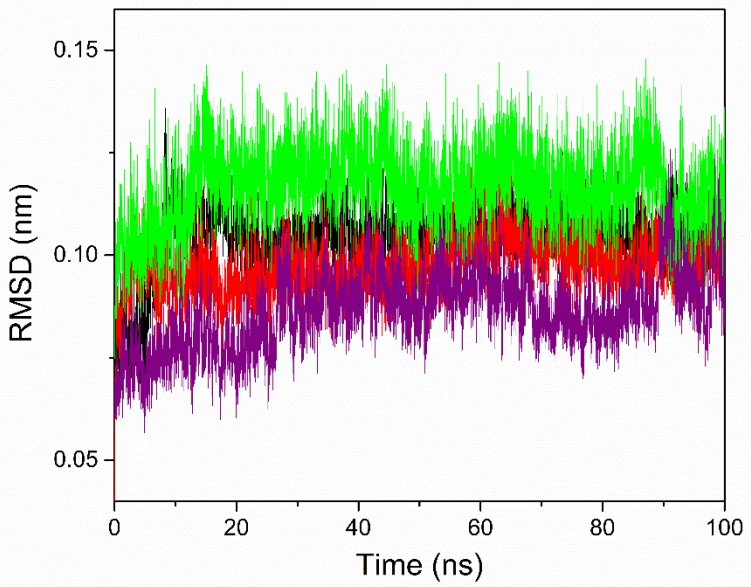


Figure 2. RMSD of trypsin solvated in pure water (black) and in the aqueous solution of Na_2SO_4 (purple), NaCl (red) and $NaClO_4$ (green) at concentration of 1M.

other salts solutions and water. In addition, RMSD shown in Figure 2, reveals that the addition of 1M solution of NaCl slightly increased the rigidity of trypsin slightly while the increase is more pronounced in the presence of Na_2SO_4. On the other hand, in the presence of chaotropic sodium anions, $NaClO_4$, the enzyme dynamics increases in comparison with its dynamics in the pure water solution. Further analyses of dynamics of the polypeptide chain and selected amino acids residues interaction with anions by root mean square fluctuation (RMSF) (Figure S1) and radial distribution function (RDF) (Figures S2, S3), respectively, are provided in the Supporting information.

Visual comparison of the global structure of the trypsin, represented by the polypeptide chain, and the local conformation of the active site of the trypsin in the presence of 1M sodium sulfate and 1M sodium perchlorate with those in the absence of salt does not reveal any significant conformational change after 100 ns simulation (Figure 3). However, a detailed analysis of MD simulations indicates that Ser217 in the S1 binding pocket and the residues

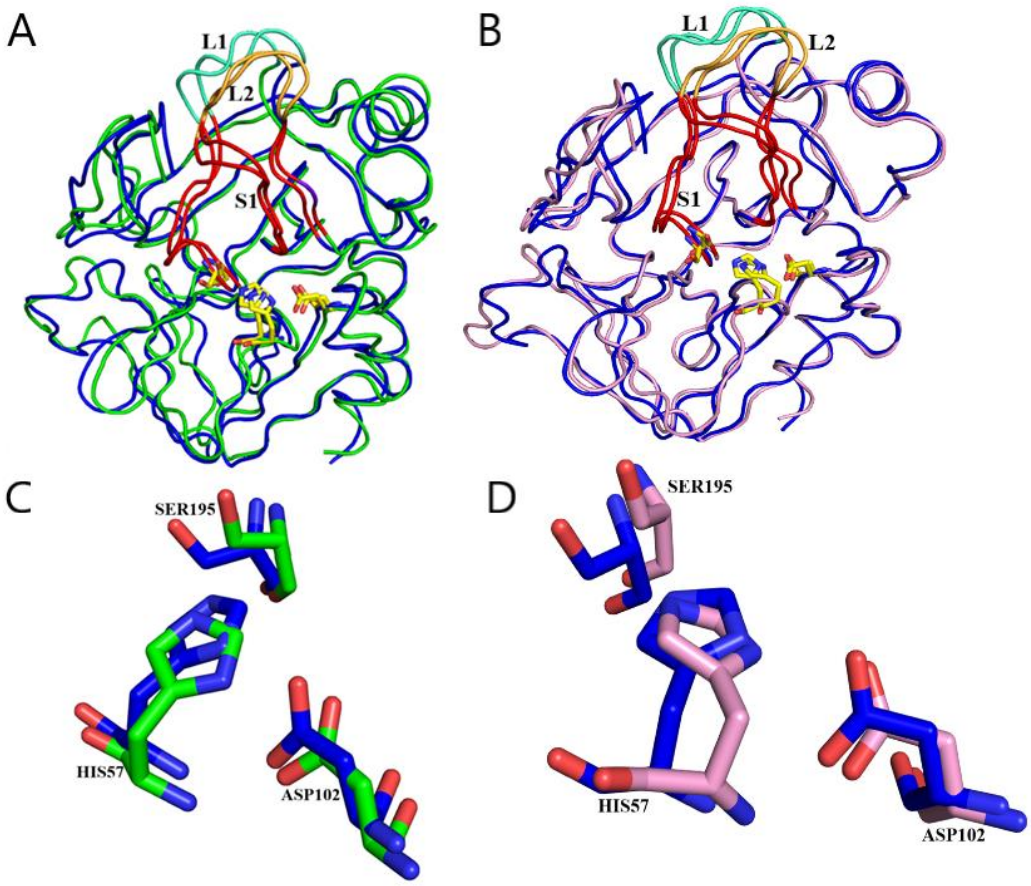
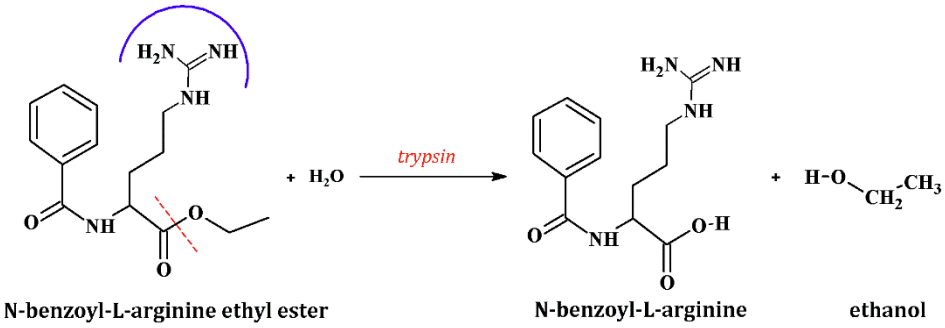


Figure 3. Superposition of trypsin (structures 100 ns after MD) in pure water (**blue**) and in the aqueous solution of Na_2SO_4 (**green**) (A, C) and $NaClO_4$ (**pink**) (B, D). Active site residues of trypsin are shown in a yellow stick representation (A, B). **Loop L1** is shown in green cyan, **loop L2** - in bright orange. **S1 binding pocket** is shown in red.

Lys222 and Lys224 in the loop L2 have increased affinity to sulfate in comparison with the perchlorate anions with possible influence on the stability of this part of the enzyme (Table S1).

Global/local property – catalytic activity. Parameters describing catalytic properties of enzymes such as k_{cat} (V_{max}) and K_M are more intriguing because they reflect both global and local properties of the enzyme in a complex way. For analysis of the catalytic properties of trypsin, we used substrate N-benzoyl-L-arginine ethyl ester (BAEE). Trypsin hydrolyzes BAEE by releasing ethanol and N-benzoyl-L-arginine as chromophore of which absorbance at 253 nm is higher than that of BAEE (Figure 4). Plotting of the initial velocities of BAEE hydrolysis on its concentration led to hyperbolic dependences described by the Michaelis–Menten equation



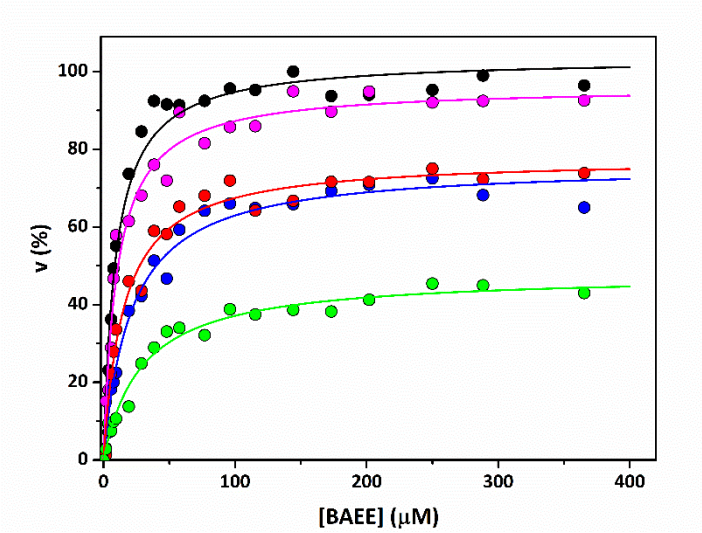


Figure 4. Schematics of the used substrate binding part (blue semi-arch) into the active site of trypsin and the cleaved bond in the substrate (red dashed line) (upper part). Lower part: Michaelis-Menten dependences of trypsin-catalyzed reaction for substrate BAEE in the absence of salt (**black symbols**), and in the presence of 1 M sodium salt; perchlorate (**green**), bromide (**blue**), chloride (**red**), and sulfate (**purple**). The experimental data (symbols) were fitted by Equation 3.

(Equation 3). The k_{cat} value for trypsin in 20 mM phosphate buffer with pH 7.6 was 15.2 s^{-1} (V_{max}=100%). For a better comparison of the obtained values in all conditions, we plotted the relative values of the initial velocities. Analysis of the fits of the experimental data by the Michaelis–Menten equation showed that both V_{max} and K_M depend on the anion type (Figure 4)

and the salt concentration (Figure S4). The parameters of the parameters V_{max} and K_M on the anion type are reversed to each other in such a way that V_{max} increases in the order of anions perchlorate<bromide<chloride<sulfate while the values of K_M decrease in the same order of the anions (Table 1).

Local property - ligand binding. For assessing of local properties of the substrate binding site, we used analysis of the dissociation constant of the fluorescent probe, p-aminobenzamidine, to trypsin in the presence of the studied salts. PAB is weakly fluorescent in neutral aqueous buffer, with excitation and emission maxima at 293 and 376 nm, respectively [48]. Binding to trypsin results in a blue shift of the emission to 363 nm and increased fluorescence. From the observation of increasing PAB fluorescence when dissolved in solvents of decreasing polarity, it has been concluded that the observed increase in PAB fluorescence upon binding to trypsin is due to hydrophobic interactions in the binding site [48]. The fluorescence emission intensity of PAB in the presence of trypsin depends on the type of anions and its concentration and increases in the order: perchlorate<bromide<chloride<sulfate (Figure S5). Dependences of the PAB fluorescence intensity in the presence of 5 μM trypsin on the PAB concentration in 1 M salts were used for determination of the dissociation constants, K_d. Obtained K_d values depend on the type of anions and they decrease in the order: perchlorate>bromide>chloride>sulfate (Figure 5, Table 1).

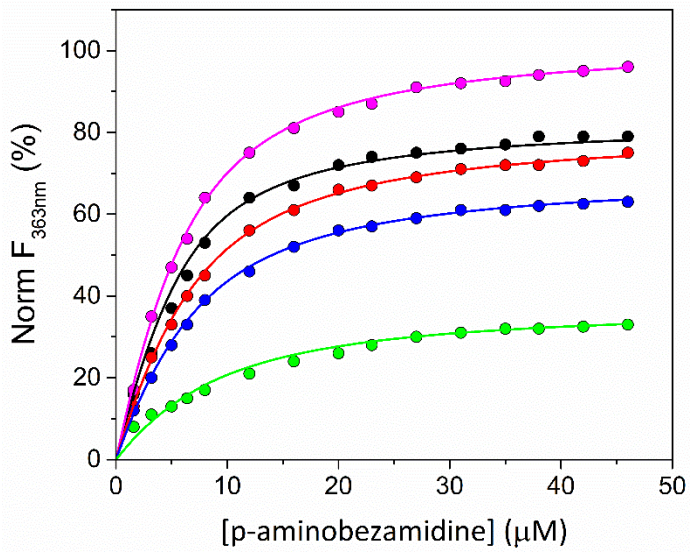


Figure 5. PAB fluorescence dependence in the presence of trypsin on the PAB concentration in the absence of salts (**black**) and in the presence of 1 M sodium salt; perchlorate (**green**), bromide (**blue**), chloride (**red**), and sulfate (**purple**). The experimental data (symbols) were fitted by Equation 2.

Plotting of the K_M values versus the maximal velocity of catalysis, V_{max}, shows linear dependence (Figure 6A) In analogy, the plot of the K_d values versus the amplitude of fluorescence intensity, F_{max}, in 1 M salts shows strong linear correlation, suggesting increased affinity of trypsin to PAB due to strengthened hydrophobic interactions in more kosmotropic anion such as sulfate in comparison with a more chaotropic anion such as perchlorate. From

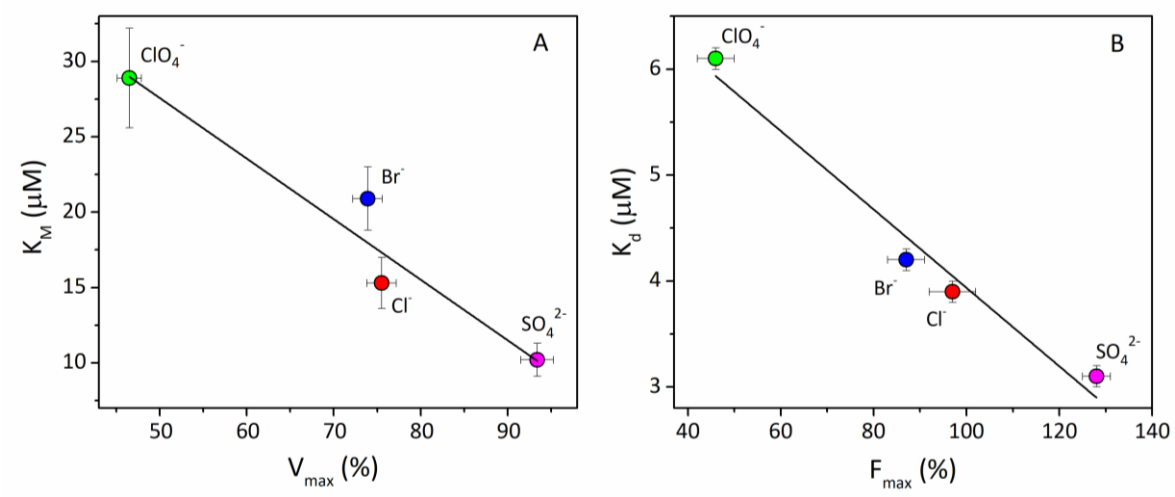


Figure 6. The correlations between K_M and V_{max} (A) and K_d and F_{max} (B) obtained in the presence of 1 M corresponding sodium anions.

both correlations in Figure 4 one can conclude, assuming analogous tendency of BAEE and PAB binding to trypsin, that tighter binding of the substrate leads to a higher catalytic rate of the substrate hydrolysis by trypsin.

Correlations of conformational and functional parameters with the intrinsic anion properties. Despite numerous works regarding how ions affect the properties of proteins/enzymes, the answer, which ion property/properties determine their effect is still unclear. The correlations between modulated enzyme properties and intrinsic properties of ions may provide an indication of relationship between the corresponding parameters. There are many parameters, which describe specific properties of ions. Fortunately, some of these parameters correlate between each other and thus significantly reduce the number of meaningful correlations. For example, the properties such as charge density, surface tension and air/water partition coefficient correlate between each other as well as viscosity B-coefficient and partition coefficient at hydrocarbon surface. In the case of trypsin, plotting of the conformational parameters represented by T_m and K_d for PAB values as well as functional parameters represented by K_M and V_{max} values versus charge density of the corresponding anions clearly show strong linear correlations (Figure 7). These correlations suggest the essential role of the electrostatic interaction of anions with trypsin in the modulation of monitored conformational

and functional trypsin parameters. In accordance with anions charge density, the strong correlations of K_M and V_{max} exist with the partition coefficient of anions for air/water interface, but no correlations with the partition coefficient of anions for hydrocarbon and polar amide surfaces (Figure S6) or polarizability of anions (Figure S7).

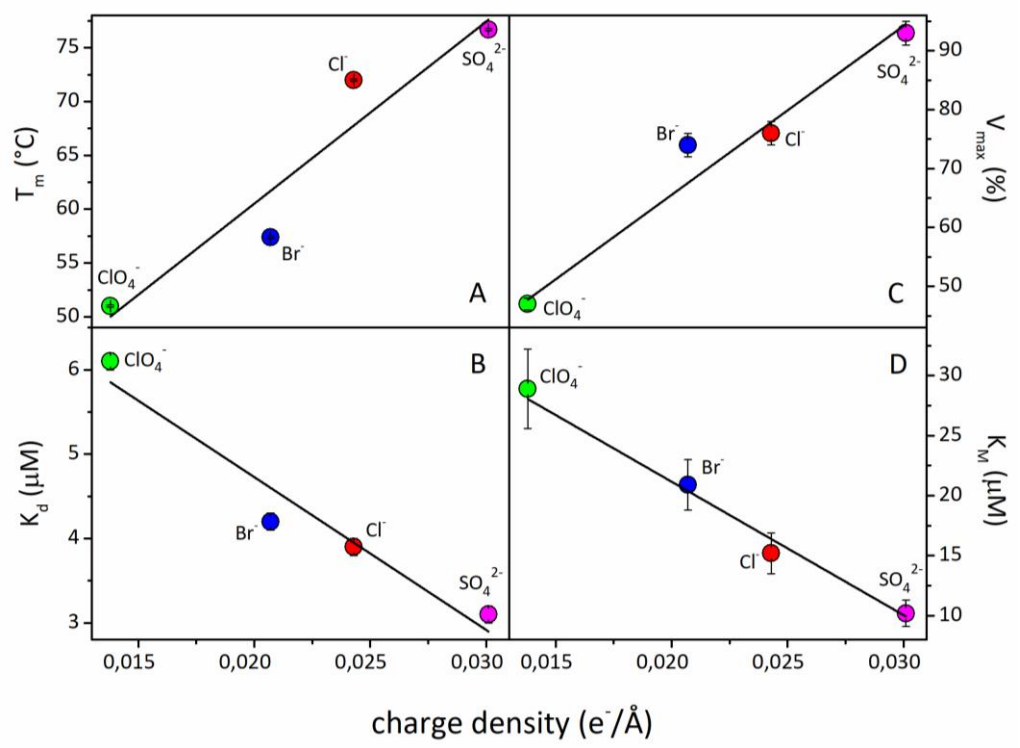


Figure 7. The correlations between parameters related to conformational properties, T_m (A) and K_d (B), and to functional properties, V_{max} (C) and K_M (D), of trypsin, which were affected by anions and the charge density of the corresponding anions.

Discussion

In the present work, we showed that anions affect global and local properties of trypsin reflected by rigidity/flexibility, thermal stability, and ligand binding, respectively. In accordance with the Hofmeister effect, the enzyme stability and its affinity for the fluorescent ligand, p-aminobenzamidine, depend on anions in the following order: sulfate>chloride>bromide>perchlorate. The same ranking can be formed to express the efficiency of anions to modulate catalytic properties of trypsin, i.e. in the presence of sulfate anions its catalytic rate is the highest and decrease in the order chloride>bromide>perchlorate while the Michaelis constants depend on anions in the reverse order, i.e. in the presence of perchlorate the K_M value is the highest and in the presence of sulfate is the lowest. Detailed analysis of these dependencies clearly suggests that the intrinsic parameter of anions that

modulate the studied properties of trypsin is the anion charge density. The examination of the intrinsic properties of anions show that the values of charge densities of studied anions correlate with partition coefficients of anions for air/water. In accordance with our previous works, we interpret such correlations as an indication of direct interaction of anions with the protein surface [13,14,49]. In fact, the effect of ions on properties of proteins through direct interaction has been demonstrated previously experimentally [50–55] and theoretically by molecular dynamics simulations [56,57]. Our conclusion regarding the direct interaction of anions determined by their charge is supported by a very recent paper by Gregory et al. [58]. The authors of this work showed that, for anions, the Hofmeister series can be explained and quantified by consideration of site-specific electrostatic interactions, approximated by the radial charge density. The interaction of anions with proteins can occur through strongly polarized peptide bond with its amino group [49,50] as well as through C_α-binding site and hydrocarbon/hydrophobic patches toward of which affinity of anions increases with their size [54]. Interestingly, although polarizability is related to the anion size, in the case of trypsin, the correlation between the polarizability of anions and the enzyme properties was not observed. This suggests that anion polarizability as a major factor governing anion/bulk water partitioning coefficients [59] is less critical for anion interaction with protein surfaces and/or depends on particular surface properties [60]. This conclusion is in accordance with several previous studies [13,14,49,61] as well as with a recent model, which takes into account both properties of ions and protein interface [62,63]. In this model, the ion specificity is explained as a result of the interplay of electrostatic and ion dispersion (van der Waals) forces.

On the other hand, there were identified close correlations between parameters describing in general unrelated properties of trypsin such as stability and catalytic properties of enzyme and affinity of small ligand into the binding site. It had been accepted that stability and conformational flexibility inversely correlate with each other [64,65], however, a more recent view indicates that the thermal stability of a protein does not necessarily correlate with the suppression of internal fluctuations of polypeptide chain [66]. In the same time, the flexibility of the polypeptide chain seems to be an inevitable factor for efficient enzyme catalysis [35,67,68]. The reaction-coupled flexibility is apparently a conserved aspect of the enzyme molecular architecture [32,34,69], which is a conclusion consistent with the macromolecular rate theory (MMRT) [36]. Central to MMRT is the decrease of the heat capacity of the enzyme-transition state in comparison with the enzyme-substrate complexes. This is not just a function of rigidification of structural elements around the active site but significant contributions are also made by regions remote from the active site [37,70]. In the present work, MD simulation

shows increased dynamics of the whole trypsin polypeptide chain but without apparent significant changes in the global protein conformation or local conformations of its active site. However, a detailed analysis of the region of the active site suggests strong interaction of anions with Ser217, forming the binding site, and particularly with Lys222 and Lys224, which are part of the loop L2.

The kinetic mechanism of serine protease reaction can be divided into three steps: enzyme-substrate complex formation, acylation and deacylation (Scheme 1). Analysis of hydrolysis of peptides by chymotrypsin, the member of the serine protease family homologous to trypsin, with different lengths showed that hydrolysis of longer peptides is more efficient than the shorter ones [71]. This effect has been interpreted as enthalpy activation intermediated through binding to distant sites in the enzyme. Detailed examination of the kinetics of the reaction suggested a significant rate increase of the acylation step with minimal effect on the complex enzyme-substrate formation and the deacylation steps. In our recent work, we showed on the example of chymotrypsin that the Hofmeister effect is substrate specific in such a way that short amide substrate (N-succinyl-L-phenylalanine-p-nitroanilide, SPNA) is significantly more activated than short ester substrate (N-benzyl-L-tyrosine ethyl ester, BTEE) by kosmotropic sulfate anions [14]. This has been interpreted as an increase in the acylation step rate in the case of SPNA in accordance with the conclusion of Zerner et al. [72] that acylation is rate determining step for amide substrate. Obtained results also suggested an importance of rigidity/stability of chymotrypsin for the acylation step in the catalytic reaction of chymotrypsin-like serine proteases. The present study, provided us with an opportunity to assess Hofmeister anions effect on the catalytic properties of the homologous serine protease – trypsin. Analogously with chymotrypsin, we observed relatively small effect of kosmotropic anions on trypsin catalytic hydrolysis of ester bond. In fact, in 1 M sulfate the value of k_{cat} for trypsin even slightly decreased (by ~7%) in comparison with trypsin catalytic rate in the absence of salt. However, in the contrast with chymotrypsin we were able to assess property of the binding site of trypsin through binding of the fluorescent probe represented by PAB. The fluorescence of PAB upon binding to trypsin clearly depends on the type of the salts, i.e. the fluorescence amplitude and the affinity of the ligand decreases from kosmotropic sulfate to chaotropic perchlorate. This suggests that the modulation of catalytic parameters, k_{cat} and K_M, of trypsin by salts is likely due to the modulation of both dissociation constant, K_d, and in analogy with chymotrypsin, possibly also the rate constant of the acylation step [72].

Specific anion effect on trypsin (this work) and chymotrypsin [14] suggest that modulation of their stabilities and rigidities affect both enzyme-substrate complex formation and likely the

acylation step. By comparing our work with the others we noticed that kosmotropic anions significantly activate enzymes undergoing an anion-triggered conformational change [18,20,73,74] and/or suboptimized reactions such as in the case of hydrolysis of amide bond by chymotrypsin [14,24]. In the case of properly folded and/or optimized reaction, kosmotropic anions either activate the enzyme only slightly or even have an inhibiting effect [6,13,14,19,22,23,76,77]. Assuming that enzymes activity is optimized in vivo environment, results obtained in in vitro conditions suggest that the enzymes are in suboptimal states regarding conformational state for catalysis of given reaction. Thus, observed effect of kosmotropic anions might mimic effect of (crowding) environment in the cells by stabilization of properly folded enzyme conformation (accompanied by significant enzyme activation) and/or by modification of polypeptide chain dynamics by increasing its rigidity (accompanied by usually only slight increase or even decrease in enzyme activity). For completion, we add that there are examples of stable properly enzymes that are activated by chaotropic agents such as chaotropic anions [4,78,79] or denaturants [68,80–83] suggesting thus importance of loosening of the enzyme structure [84] and existence of optimal flexibility of the reaction site in enzymes in accordance with a hypothesis of a "corresponding state" regarding conformational flexibility [6,67,68].

Conclusion

We show that the specific anion effect influences the global property of trypsin represented by its thermal stability and at the same time its local properties, which can be assessed by ligand binding parameters. In consent with that, the catalytic parameters, which are according to the macromolecular rate theory affected by both global and local conformational properties of the enzyme, correlate with the modulated global and local properties of trypsin. Moreover, correlations of the modulated enzyme properties with the intrinsic properties of the studied anions suggest a critical role of charge density for specific anions effect on trypsin. We interpret such correlations as an indication of direct interaction of anions with the protein surface. Analysis of the catalytic parameters of trypsin in the presence of salts strongly indicates the effect of anion on the formation of the enzyme-substrate complex and possibly the acylation step through affecting dynamics of distant sites on the enzyme.

CRediT authorship contribution statement

Eva Dušeková, Katarína Garajová, Rukiye Yavaşer, Dagmar Sedláková, Veronika Dzurillová, Natalia Kulik, Anastasiia Shaposhnikova, Fatemeh Fadaei: Investigation,

Formal analysis **Mária Tomková**: Writing – Review & Editing **Babak Minofar**: Conceptualization, Writing – Review & Editing **Erik Sedlák**: Funding acquisition, Formal analysis, Writing – Original Draft, Review & Editing.

Declaration of competing interest

None.

Acknowledgements

This work was supported by Slovak Research and Development Agency through the project APVV-20-0340 and by the grant agency of the Ministry of Education, Science, Research, and Sport of the Slovak Republic (grant no. VEGA 1/0074/22) and by the EU H2020-WIDESPREAD-05-2020 grant No. 952333, CasProt (Fostering high scientific quality in protein science in Eastern Slovakia). This publication is the result of the project implementation: Open scientific community for modern interdisciplinary research in medicine (Acronym: OPENMED), ITMS2014+: 313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF. Babak Minofar thanks computational resources which were supplied by the project "e-Infrastruktura CZ" (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures. In addition, B.M acknowledges grant GAČR 21-15936S from the Czech Science Foundation.

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